Supplementary Material

Supplementary Figures

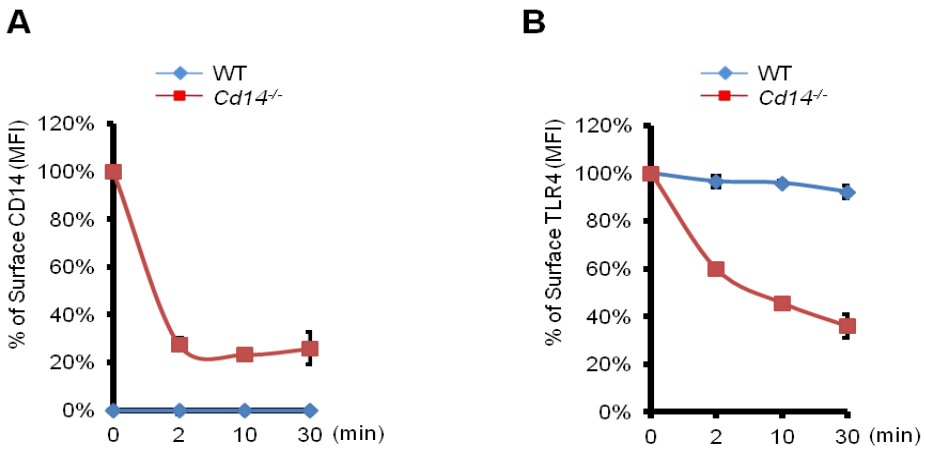

Figure S1 Tan et al

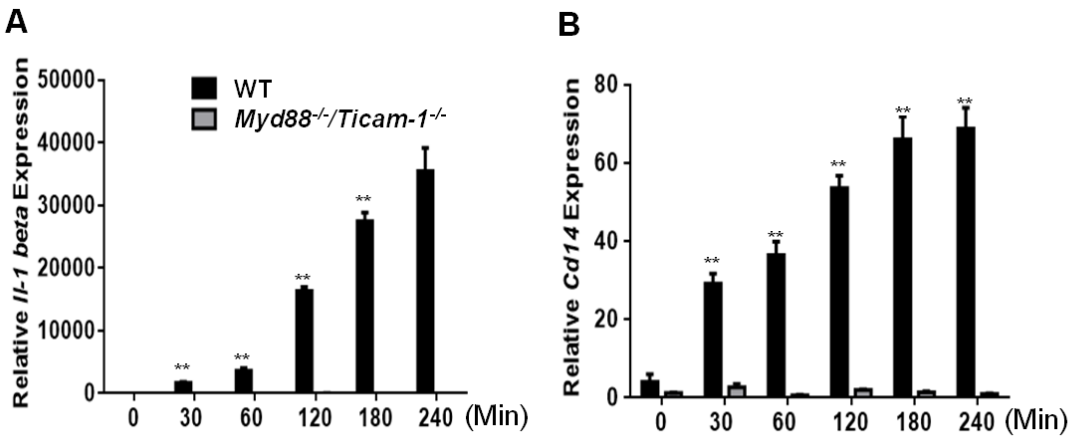

Figure S2 Tan et al

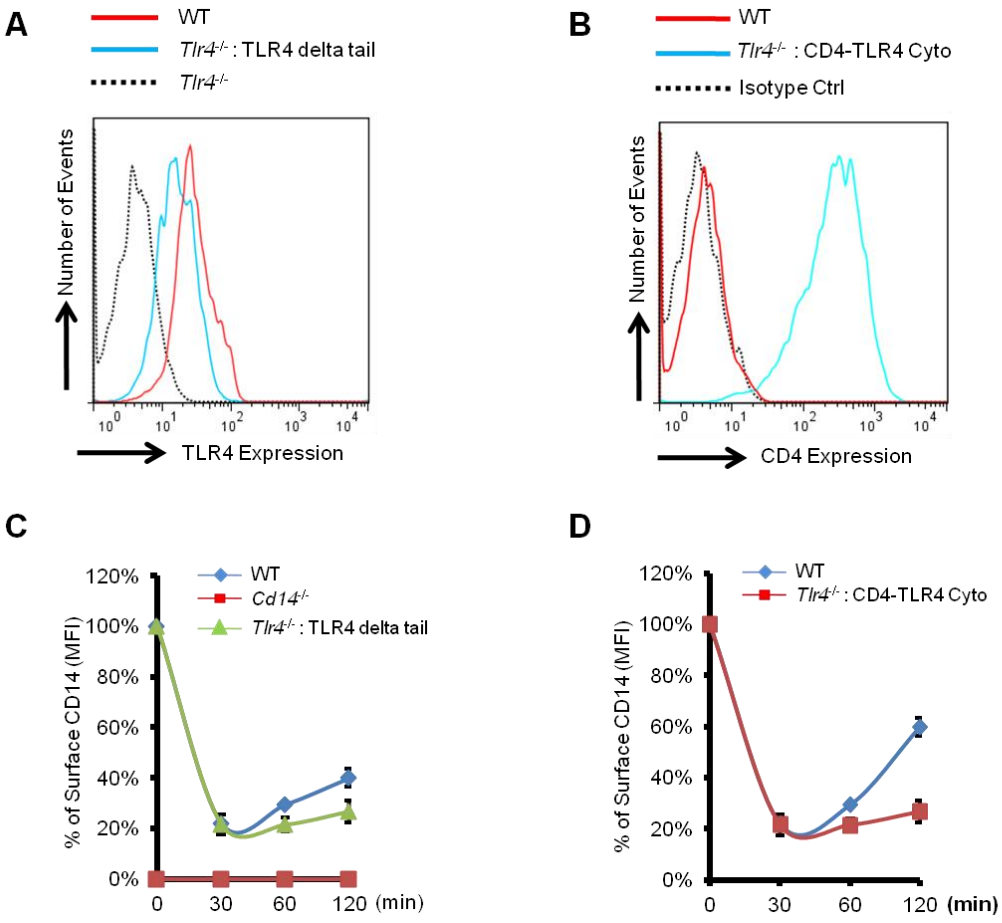

Figure S3 Tan et al

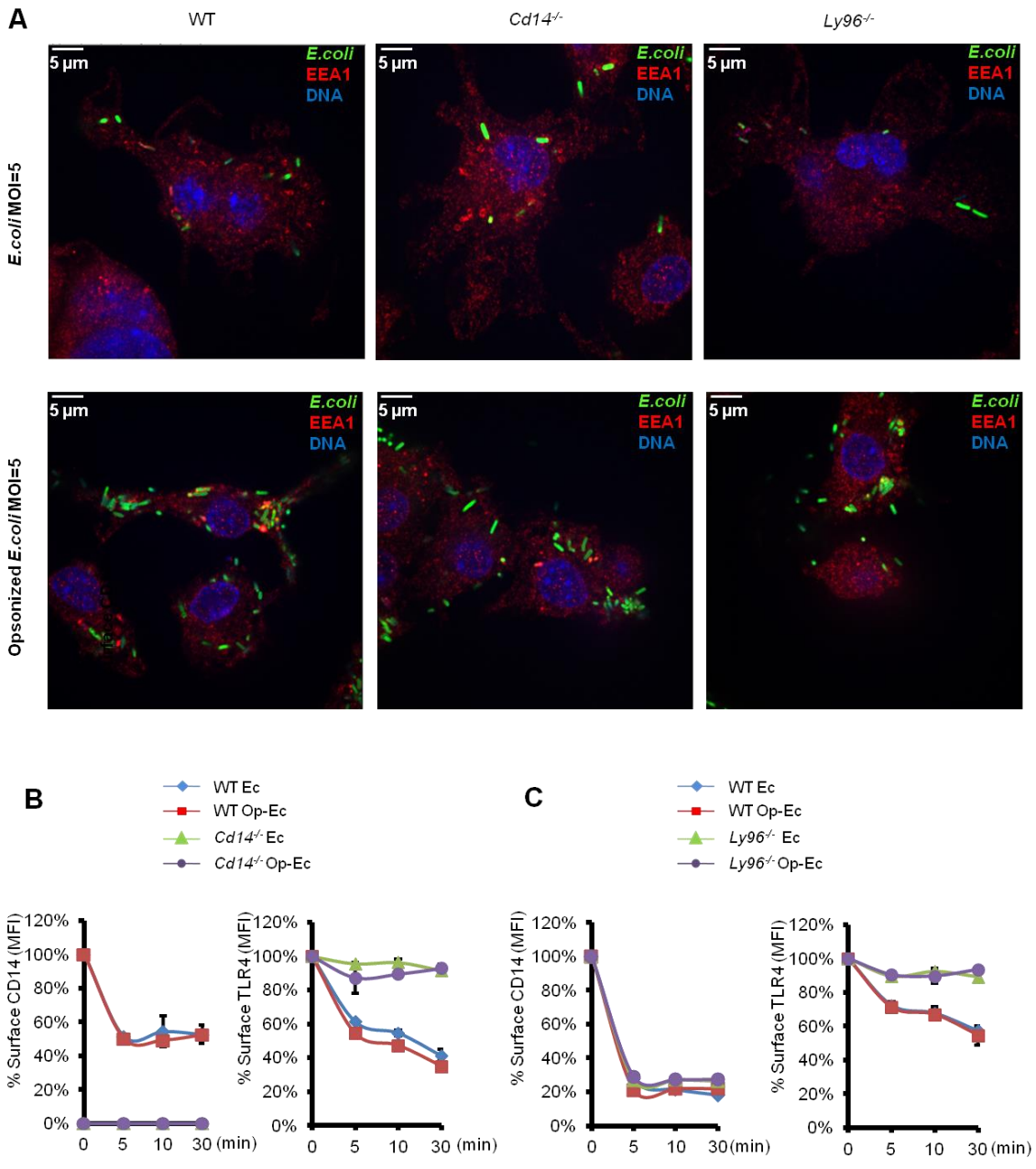

Figure S4 Tan et al

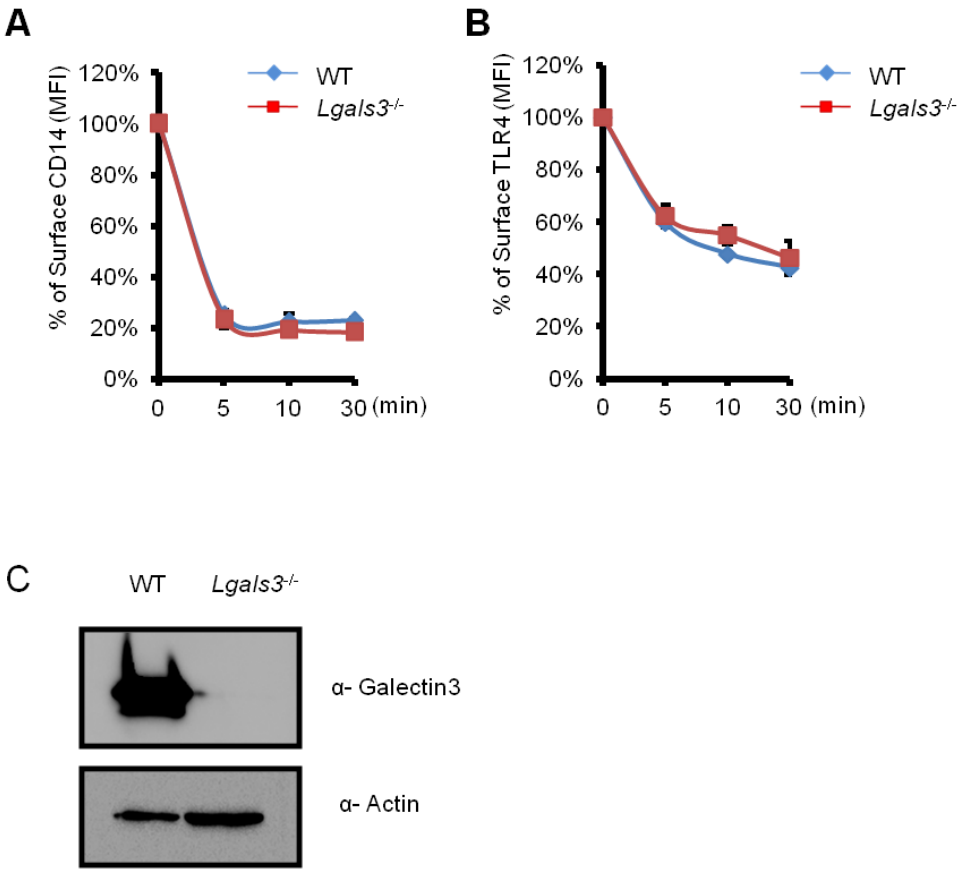

Figure S5 Tan et al

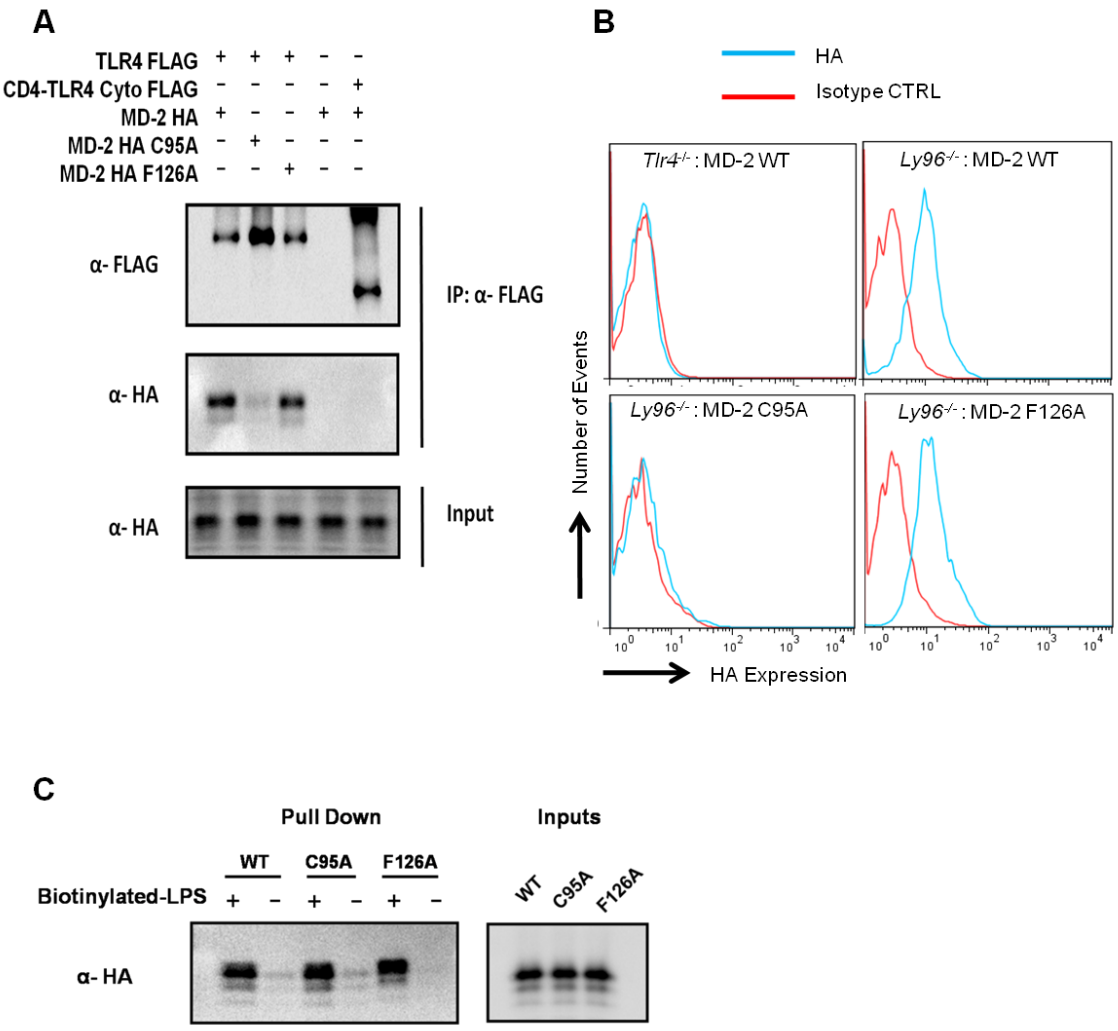

Figure S6 Tan et al

Supplementary Figure Legends

Figure S1. LPS-induced Receptor Endocytosis Occurs in a Short Time Frame (related to Figure 1)

(A) Surface level of CD14 was monitored by flow cytometry in WT and *Cd14*$^{-/-}$ iBMDMs stimulated with LPS at indicated time points.

(B) Surface level of TLR4 was monitored by flow cytometry in WT and *Cd14*$^{-/-}$ iBMDMs.

All FACS experiments were performed three times and one representative result out of three is presented. Error bars represent mean SEM from triplicate readings in one experiment.

Figure S2. The Upregulation of *Cd14* mRNA Transcription is Dependent on NF-κB signaling (related to Figure 2)

(A) Time dependent *Il1b* expression in WT and *Myd88*$^{-/-}$/*Ticam1*$^{-/-}$ iBMDMs. Cells were stimulated with LPS at indicated time points. The expression of *Il1b* was measured by qPCR.

(B) Time dependent Cd14 expression in WT and *Myd88*$^{-/-}$/*Ticam1*$^{-/-}$ iBMDMs. Cells were stimulated with LPS at indicated time points. The expression of *Cd14* was determined by qPCR.

qPCR data shown were representative data from at least three independent experimnets. Error bars represent mean SEM from triplicate readings in one experiment. **,$p<0.01$

Figure S3. Expression of Different TLR4 Alleles on *Tlr4*$^{-/-}$ iBMDMs (related to Figure 3)

(A)Surface level of TLR4 was determined by flow cytometry in WT iBMDMs and *Tlr4*$^{-/-}$ iBMDMs expressing the TLR4-Tailless allele. *Tlr4*$^{-/-}$ iBMDMs were stained as negative control.

(B) Surface level of CD4 was determined in the indicated cell lines

(C) Cells with the indicated genotypes were treated with LPS, and the efficiency of CD14 endocytosis was measured by flow cytometry. Line graph represents the MFI of CD14 surface staining at different time points from the indicated cell lines. *Cd14*$^{-/-}$ iBMDMs were used as negative control.

(D) WT and *Tlr4*$^{-/-}$ iBMDMs expressing the CD4-TLR4 Cyto allele were treated with LPS, and surface level of CD14 was determined by flow cytometry.

All FACS experiments were performed three times and one representative result out of three is presented. Error bars represent mean SEM from triplicate readings in one experiment.

Figure S4. The Cargo Selection Role of MD-2 for TLR4 Endocytosis is Independent on the Cargo Properties and the Means of Entry (related to Figure5)

(A) Microscopic images demonstrated that antibody-mediated opsonization enhanced uptake of GFP-*E.coli* by iBMDMs. iBMDMs of the indicated genotypes were infected with GFP-*E.coli* (upper) and opsonized GFP-*E.coli* (lower) at an MOI of 5. Infection was allowed to proceed for 5 min and unbound bacteria were washed off. Cells were then fixed and early endosome antigen 1 (EEA1) and nucleus DNA were stained as indicated.

(B) Flow cytometry analysis of CD14 endocytosis and TLR4 endocytosis in *E.coli* infected WT and *Cd14*$^{-/-}$ iBMDMs. Cells were infected with *E.coli* (untreated and opsonized) at an MOI of 100 for the indicated time points. Surface levels of CD14 (left) and TLR4 (right) were monitored by flow cytometry.

(C) Flow cytometry analysis of CD14 endocytosis and TLR4 endocytosis in *E.coli* infected WT and *Ly96*$^{-/-}$ iBMDMs. Cells were infected with *E.coli* (untreated and opsonized) at an MOI of 100 for the indicated time points. Surface levels of CD14 (left) and TLR4 (right) were monitored by flow cytometry.

Microscope images were selected fields out of 150 fields examined, and >90% of the cells displayed similar staining pattern. All FACS experiments were performed three times and one representative result out of three is presented. Error bars represent mean SEM from triplicate readings in one experiment.

Figure S5 LPS-induced Receptor Endocytosis is Independent of Galectin-3 (related to Figure 4)

(A) Surface level of CD14 was monitored by flow cytometry in WT and *Lgals3*$^{-/-}$ primary BMDMs stimulated with LPS at indicated time points.

(B) Surface level of TLR4 was monitored by flow cytometry in WT and *Lgals3*$^{-/-}$ primary BMDMs.

All FACS experiments were performed three times and one representative result out of three is presented. Error bars represent mean SEM from triplicate readings in one experiment.

(C) Detection of Galectin3 expression in WT and *Lgals3*$^{-/-}$ primary BMDMs by western blotting

Figure S6. Biochemical and Cell Biological Properties of the Selected MD-2 Mutant Alleles (Refers to Figure 5).

(A) 293T cells were transfected with various MD-2, TLR4 constructs in the indicated combinations. 48 hours after transfection, cells were lysed and Flag-tagged TLR4 were immunorecipitated by M2-conjugated resin, the isolated protein complexes were eluted by FLAG peptide. The eluates were concentrated by TCA precipitation and boiled with SDS loading buffer. Proteins were separated on 4-20% gradient SDS PAGE gels, transferred on nitrocellulose membranes and visualized by western blotting.

(B) Surface staining of MD-2 alleles on *Tlr4*$^{-/-}$ and *Ly96*$^{-/-}$ iBMDMs. HA-tagged MD-2 alleles were expressed in *Tlr4*$^{-/-}$ and *Ly96*$^{-/-}$ iBMDMs by retroviral transduction. Surface MD-2 were stained with APC conjugated HA (APC anti-HA) antibody and detected by flow cytometry.

(C) In vitro LPS pull down assay with different MD-2 alleles. 293T cells were transfected with indicated MD-2 constructs. 48 hours after transfection, cells were lysed, and 5 μg of biotinylated LPS were added into the lysates. The lysates and biotinylated LPS were incubated at 4°C for 2 to 3 hours, and streptavidin conjugated resin were added to isolate the MD-2-LPS complex. LPS associated MD-2 were detected by western blot using an anti-HA antibody.

Protein blots shown were representative data from at least three independent experiments. All FACS experiments were performed three times and one representative result out of three is presented. Error bars represent mean SEM from triplicate readings in one experiment.